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Neutralization of Bacterial Lipopolysaccharides by Human Plasma

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To quantify the neutralization of bacterial lipopolysaccharide (LPS) by human plasma, dilutions of *Escherichia coli* O113 LPS were incubated with plasma, followed by the addition of *Limulus* amebocyte lysate (LAL). The reaction between the LPS and LAL was monitored spectrophotometrically, and the concentration of LPS resulting in 50% lysate response (LR_{50}) was determined. Analysis of 145 outdated plasma samples yielded a range of LR_{50} between 6 and 1,500 ng/ml. Pools of plasma with high and low LR_{50} were prepared. The pool with high LR_{50} neutralized 166-fold more *E. coli* 0113 LPS, 190-fold more *E. coli* 0111B4 LPS, 42-fold more *Klebsiella pneumoniae* LPS, and 29-fold more *Salmonella typhimurium* LPS than did the pool with low LR_{50}. Each pool had similar immunoglobulin G (IgG) and IgM antibody levels to homologous LPS, measured by an enzyme-linked immunosorbent assay. Analysis of 212 fresh-frozen plasma units revealed a range of LR_{50} between 48 and 6,000 ng/ml. Incubation of LPS in a pool of fresh-frozen plasma with high LR_{50} elicited significantly less fever in the rabbit pyrogen test than did LPS incubated in plasma with low LR_{50} (fever index, 2.68 ± 0.61°C · h and 3.52 ± 0.66°C · h, respectively; $P = 0.003$). We conclude that there is a 100-fold range in the endotoxin-neutralizing capacity of human plasma and that this variation is not due to LPS-specific IgG or IgM antibodies. Further investigations are needed to determine whether differing susceptibility of patients to the effects of LPS is due to differences in the endotoxin-neutralizing capacity of their plasma and whether plasma screened for high endotoxin-neutralizing capacity may be therapeutically useful in endotoxemia.

Bacterial lipopolysaccharide (LPS) is important in the pathogenesis of gram-negative infections. LPS has many potent biological activities including induction of fever, hypotension, and disseminated intravascular coagulation. Human and animal plasma inhibit the biological activities of LPS in a variety of systems including tumor necrosis (5), rabbit pyrogenicity (12, 17), chicken embryo death (13), and death of Thorotrast-treated mice (17). Human serum and plasma also inhibit the ability of LPS to gel *Limulus* amebocyte lysate (LAL), a phenomenon that has hampered investigators attempting to use the LAL test to detect circulating LPS (6, 15). In this study we examined the variability in endotoxin-neutralizing capacity of plasmas from healthy blood donors by using inhibition of LAL gelation as an assay.

Plasmas screened for high neutralizing capacity by this assay were then compared with plasmas screened for low neutralizing capacity in their ability to inhibit LPS-induced fever in rabbits. Neutralization of the biological activity of LPS may be an important factor in host resistance to LPS challenge. An understanding of the mechanisms of LPS detoxification in plasma may lead to methods for the identification of specific plasma units or purified plasma fractions with therapeutic usefulness in the treatment of gram-negative sepsis.

MATERIALS AND METHODS

LPSs. *Escherichia coli* 0113 LPS was obtained from Associates of Cape Cod, Inc., Woods Hole, Mass. LPSs from *E. coli* 0111 B4, *Klebsiella pneumoniae*, *Salmonella typhimurium*, *S. minnesota* Re 595, and *E. coli* J5 were obtained from List Biological Laboratories, Campbell, Calif.

Plasma. Samples of units of human outdated plasma stored at 4°C for 14 to 50 days were obtained from Jeanne Leszczynski, Massachusetts Public Health Laboratories, Boston, Mass. Fresh-frozen plasma was kindly provided by Peter Page, Northeast Region American Red Cross, Needham, Mass. All plasma samples were collected in bags containing anticoagulant citrate phosphate dextrose solution USP.

Antibody testing. Human immunoglobulin G (IgG)- and IgM-class antibodies to specific LPS were measured by ELISA; the method of Engvall (2) was used. Flexible polyvinyl U-bottomed 96-well microtiter plates (no. 001-010-2401; Dynatech Laboratories, Inc., McLean, Va.) were coated with 100 μl of LPS per well at a concentration of 1 μg/ml in phosphate-buffered saline (PBS; 1 mM $NaH_2PO_4 \cdot H_2O$, 5 mM $Na_2HPO_4 \cdot 7H_2O$, 138 mM NaCl, 15 mM NaN_3 [pH 7.2]). After incubation for a minimum of 18 h at 4°C, the plates were washed three times with wash buffer (PBS, 0.02 M $MgCl_2$, 0.05% [vol/vol] Tween 20). Sample (in PBS-bovine serum albumin) (100 μl) was added to each well and incubated overnight at 4°C. After the wells were washed three times with wash buffer, 100 μl of gamma-chain-specific alkaline phosphatase-conjugated goat anti-human IgG and mu-chain-specific goat anti-human IgM (Cooper Biomedical, Malvern, Pa.) at a concentration of 0.2 μg of goat IgG in PBS-bovine serum albumin was added and incubated at 37°C for 2 h. Plates were washed twice with wash buffer and once with DEA buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, 3 mM NaN_3 [pH 9.8]). The plates were developed with 100 μl of *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.) per well at a concentration of 1 mg/ml in DEA buffer. After incubation of the wells at room temperature for 40 min,

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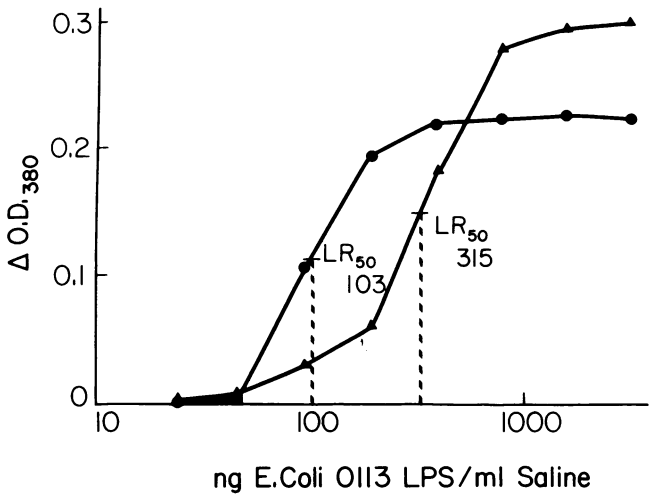

FIG. 1. Sample curves illustrating the method of calculation of LR_{50} for two human plasma samples.

50 μl of 3 N NaOH was added to each well to stop the reaction. The optical density at 405 nm (OD_{405}) was determined on a model EL3071P ELISA reader (Biotek, Burlington, Vt.).

The ELISA was standardized by the method Zollinger and Boslego (21) to permit conversion of OD to nanograms of antibody per milliliter.

Briefly, a human IgG of IgM ELISA was performed by sensitizing the wells with an IgG or IgM fraction of goat anti-human IgG or IgM (Cappel Laboratories) diluted 1:1,000 for IgG and 1:250 for IgM in PBS, adding known concentrations of IgG or IgM (Sigma Chemical Co.) varying from 10 to 1,000 ng/ml to sensitized wells, and developing them as described above. In parallel on the same plate, an ELISA for anti-LPS antibody was performed. The concentrations of IgG or IgM class anti-LPS antibody in the pool were equated with the concentration of rabbit IgG or IgM giving an equal OD in the rabbit IgG or IgM ELISA. All assays were done in duplicate, and means are given.

Protein measurement. Total protein was measured by the method of Lowry et al. (7).

LAL assay. The LAL assay was performed as previously described (11). Briefly, 50 μl of plasma sample, diluted 1:1 in normal saline (0.9% sodium chloride USP for injection; Abbott Laboratories, North Chicago, Ill.) was added to 50 μl of serial twofold dilutions of *E. coli* 0113 LPS in normal saline in 96-well microtiter plates (Flow Laboratories, McLean, Va.). After incubation for 3 h at 37°C, 100 μl of reconstituted LAL (spectrophotometric lot 265; Associates of Cape Cod) was added and mixed. After further incubation at 37°C for 1 h, the plates were read on a microplate reader (Flow Laboratories) at 380 nm. These results were plotted, and the concentration of LPS needed to produce 50% of the maximal increase in OD produced by an excess of LPS was determined by interpolation (Fig. 1). This value was called the 50% *Limulus* gelation response (LR_{50}). To control for plate-to-plate and day-to-day variation, each plate also contained a single replicate sample that had been aliquoted and frozen at −60°C. To control against variation in LPS dilutions, an assay of LPS potency was done in parallel on each plate by incubating LPS dilutions with normal saline instead of plasma. Thus, each plate contained six plasma aliquots, the single-sample control, and the LPS control.

Pyrogen tests. Pyrogen tests were done on rabbits at the Massachusetts Public Health Biologic Laboratories, Boston, Mass., in a temperature- and humidity-controlled room. New Zealand White rabbits, weighing approximately 2 kg each, were obtained from ARI Breeding Laboratories, E. Bridgewater, Mass. Rabbits were sham tested on the first

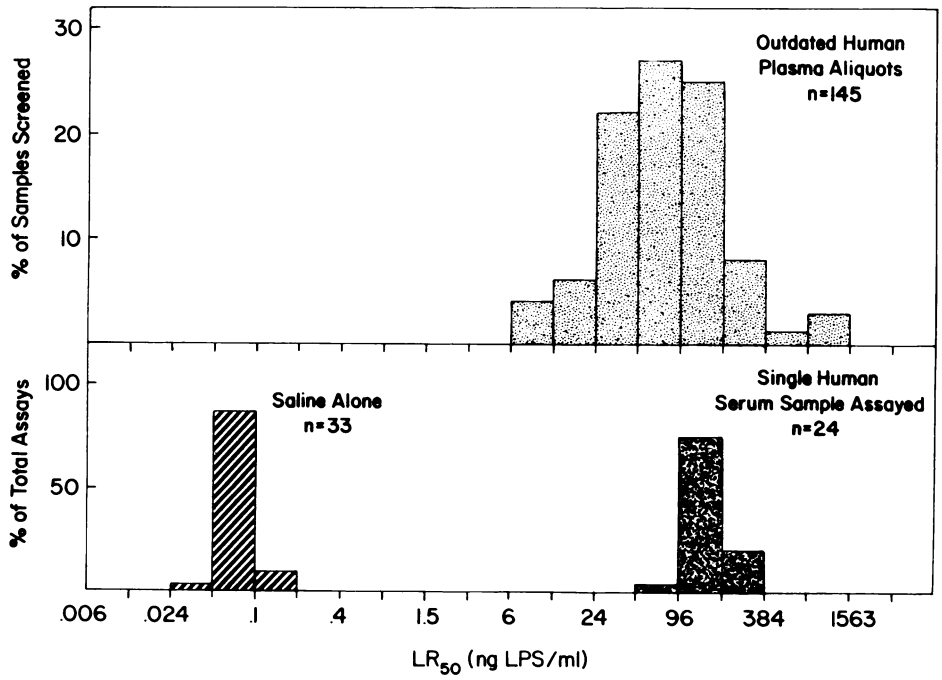

FIG. 2. Results of screening 145 recovered human plasma samples for their ability to neutralize *E. coli* 0113 LPS. The lower panel shows reproducibility of the assay in saline and human serum.

day by placing them in stocks, shaving their ears, and recording their rectal temperature hourly for 4 h with electronic probes (Telethermometer; Yellow Springs Instrument Co., Yellow Springs, Ohio). On day 2, base-line temperatures were established, the preparation to be tested was injected intravenously in the marginal ear vein, and temperatures were measured hourly for 4 hours. The fever index was calculated by adding the total number of degrees centigrade above the base-line temperature at the four hourly readings.

RESULTS

The results of screening 145 outdated plasma samples (recovered plasma) for the LR_{50} are shown in Fig. 2. Human serum and plasma inhibited the test assay approximately 1,000-fold compared with saline. Human recovered plasma varied more than 100-fold in LR_{50}. The saline and replicate sample control indicated that the assay itself was reproducible.

To further characterize the differences between plasmas with high and low LR_{50}s, the four plasma units with the highest and lowest LR_{50}s were pooled to form a high- and a low-activity plasma pool. The protein concentrations of the two pools were 5.8 and 6.1 mg/dl, respectively. The assay was repeated with samples of the pools with the LPS used for screening (*E. coli* 0113) and three heterologous smooth LPSs. The high-activity pool was able to neutralize significantly more *E. coli* 0113 LPS than was the low-activity pool, and this extended to the LPSs from three other smooth, gram-negative organisms. In addition, IgG and IgM antibody to the corresponding LPSs and to LPSs from rough mutants *E. coli* J5 and *S. minnesota* Re595 were measured in the two pools by ELISA to examine whether the difference seen reflected differences in natural antibody (Table 1). In all cases the high-activity pool contained similar IgG- and IgM-class antibody concentrations directed to the smooth or rough mutant LPSs.

The effect of incubation between LPS and plasma on the LR_{50} was examined by incubating samples of plasma from the high- and low-activity pools for various times with *E. coli* 0113 LPS at 37°C before LAL was added to the system. LR_{50}s were determined from the curves that were generated and were then plotted against incubation time (Fig. 3). At all time points examined, the LR_{50} of the high-activity pool exceeded that of the low-activity pool, and this difference increased with time to 400-fold after 180 min of incubation.

TABLE 1. Comparison of LPS LR_{50} and anti-LPS antibodies in plasma pools with low and high neutralizing activity for *E. coli* O113 LPS

LPS source	LR_{50} (ng of LPS/ml) in:		IgG antibody[a] (ng/ml) in:		IgM antibody[a] (ng/ml) in:	
	Low[b]	High[b]	Low	High	Low	High
E. coli O113	15	>2,500	5,700	2,700	400	325
E. coli O11 B4	13	>2,500	3,300	2,050	350	275
K. pneumoniae	12	500	2,700	2,300	325	400
S. typhi	6.4	200	2,200	1,700	412	450
E. coli J5	ND[c]	ND	1,500	800	237	212
S. minnesota Re 595	ND	ND	2,150	400	275	225

[a] To corresponding LPS.
[b] Low, Low-activity pool; high, high-activity pool.
[c] ND, Not determined.

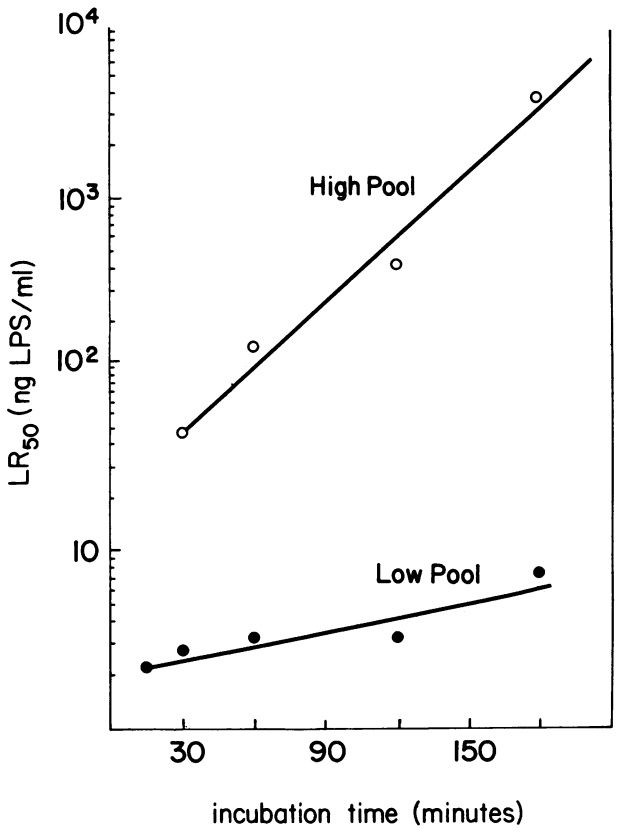

FIG. 3. Effect on LR_{50} of varying incubation time between LPS and plasma pools with low or high neutralizing ability. LPS and plasma were incubated at 37°C for the times shown, and LAL was then added. OD was measured 1 h after addition of LAL for all points.

To exclude the possibilities that plasma directly inhibited the lysate cascade by a nonspecific mechanism such as enzymatic degradation, we performed parallel experiments in which the lysate was incubated with 50% plasma in saline for various periods (0 to 180 min) followed by addition of LPS. The LR_{50} was identical to that observed without preincubation at all time points, indicating that the ability of the lysate to gel was unaltered by preincubation with plasma.

To eliminate the possibility that some of the factors responsible for LPS neutralization were lost with storage at 4°C, we screened fresh-frozen plasma samples. A total of 212 frozen segments of the tubing used to fill bags of fresh-frozen plasma were separated from the fresh-frozen units, coded, and screened by using the same methods as described above. Fresh-frozen plasma units were stored at −70°C. A single plasma unit chosen as a plasma control was thawed, divided into 1-ml aliquots, and then refrozen. This plasma was run in parallel on each plate as above. The results are shown in Fig. 4. Again, a range of LR_{50}s was found, and the entire range was approximately eightfold higher with fresh-frozen plasma than with plasma kept at 4°C for 15 or more days.

To study the lability of the factor(s) responsible for a high LR_{50}, pools were made from the 10 fresh-frozen plasma units with the highest and the 10 with the lowest neutralizing activity of the 212 units illustrated in Fig. 4. Samples of these high- and low-activity pools were removed from the −70°C freezer and stored for different periods at 4°C. The samples were then assayed in parallel with a sample of freshly thawed

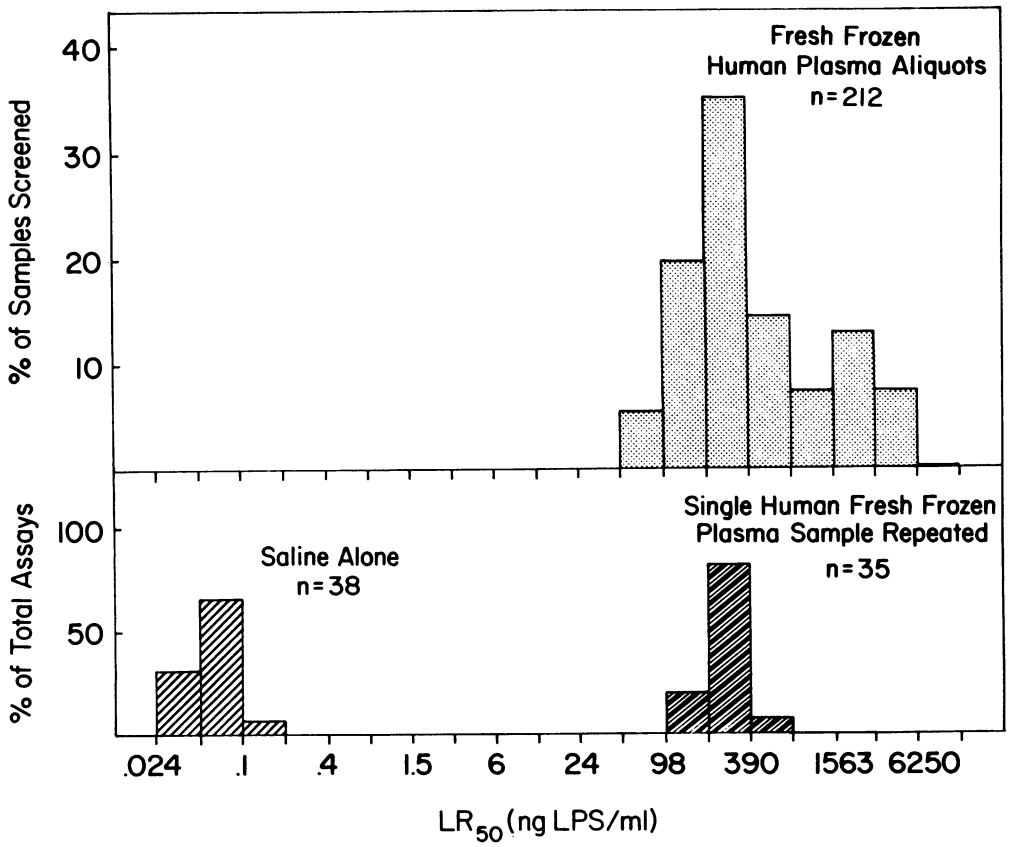

FIG. 4. Results of screening 212 fresh frozen plasma samples for ability to neutralize *E. coli* 0113 LPS. The bottom panel shows reproducibility of the assay in saline and human plasma. Note, in comparison with Fig. 2, that the entire range is eightfold higher for fresh-frozen plasma.

plasma. The results (Table 2) indicate that the pool screened for high neutralizing activity is labile at 4°C. Other experiments indicated that the LR_{50} of the high-activity pool fell to that of the low-activity pool after storage for 8 weeks at −10°C but is stable when stored at −70°C (data not shown).

To evaluate the findings of the LAL test in another system, we compared the ability of high- and low-activity plasma pools to neutralize the pyrogenicity of LPS in rabbits. We chose this model because it is well characterized and easily quantified. A dose response curve of fever induced by LPS was determined, and a dose of 150 ng of *E. coli* 0113 LPS per kg was found to be on the sensitive steep portion of the curve. LPS concentrations of 300 ng/ml were then prepared in saline or 80% plasma from each of the two fresh-frozen plasma pools, incubated at 37°C for 2 h, and injected into rabbits at a dose of 0.5 ml/kg. The results (Table 3) reveal that LPS incubated in either plasma pool elicited significantly less fever than did LPS incubated in saline. In addition LPS incubated in the plasma pool screened for high LR_{50} elicited significantly less fever than did LPS incubated in the plasma pool screened for low LR_{50} ($P = 0.003$).

TABLE 2. Lability of LR_{50} with storage of fresh frozen plasma pools at 4°C

Sample	LR_{50} (ng/ml) after days at 4°C[a]:				
	0	2	7	15	31
Low-activity plasma pool[b]	89	140	165	141	56
High-activity plasma pool[b]	15,000	7,400	4,300	5,100	52
Control plasma (assayed fresh)	280	220	200	270	305
Saline	0.06	0.07	0.05	0.05	0.06

[a] LR_{50} for *E. coli* 0113 LPS.
[b] Plasma was removed from −70°C and kept at 4°C for various times.

DISCUSSION

These studies show that there is considerable variation in the ability of human citrated plasma from healthy blood

TABLE 3. Fever index in rabbits after intravenous injection of 150 ng of *E. coli* 0113 LPS per kg incubated in saline or fresh frozen plasma pools screened for high or low LR_{50}[a]

Incubation medium	No. of rabbits	Mean fever index ± SD (°C · h)		
Normal Saline	15	4.52 ± 1.23	$P = 0.012$	$P = 0.0001$[b]
Plasma pool (low LR_{50})	13	3.52 ± 0.66	$P = 0.003$	
Plasma pool (high LR_{50})	13	2.68 ± 0.61		

[a] Solutions were incubated for 2 h at 37°C before injection by two-tailed *t* test.
[b] Comparisons by two-tailed *t*-test.

donors to neutralize smooth LPS and that this variation is not due to IgG or IgM antibody directed to the corresponding LPS or to LPS from rough mutant organisms *E. coli* J5 or *S. minnesota* Re595. LPS incubated in a pool prepared from plasma screened for high neutralizing activity by LAL gave significantly less fever when injected into rabbits than did LPS incubated in a pool prepared from plasma screened for low neutralizing activity.

Several lines of evidence suggest that the inhibition of the LAL test by plasma reflects the neutralization of the biological effects of LPS by plasma rather than an interaction between plasma and LAL. First, incubation of LAL with plasma for various periods up to 180 min before addition of LPS dilutions does not alter the LR_{50}, implying that plasma did not interfere with the *Limulus* cascade over that time period. Second, increasing the incubation time of the LPS dilutions with the pool high in neutralizing activity while keeping the LAL incubation time constant resulted in more neutralization (Fig. 3). Third, a pool made of human plasma selected solely on the basis of its ability to inhibit the LAL reaction with LPS was able to detoxify significantly more LPS in another biological model, rabbit pyrogenicity, than was a similar pool of plasma selected for its ability to provide less inhibition of the LAL test.

The mechanism of detoxification that we are measuring is not addressed in these studies. Our data, however, suggest that "natural" antibody is not responsible for the neutralization seen. Although antibody to the polysaccharide O chains of LPS is protective in animal models (1, 3, 8) and neutralizes LPS in the LAL inhibition assay (18; unpublished observations), the lack of correlation with specific anti-LPS antibodies suggest that the detoxification is not mediated by type-specific antibody. Several studies have indicated that antibody directed to a common core element of LPS, core glycolipid, is protective for a variety of smooth heterologous LPSs (9, 19, 20), yet the detoxification seen in our study did not correlate with IgG or IgM antibody to LPSs from rough mutants *E. coli* J5 or *S. minnesota* Re 595, each of which displays core glycolipid. The lability of the plasma inhibitor at 4°C provides further evidence that it is not an immunoglobulin. It is more likely that non-antibody-mediated mechanisms which are not LPS serotype specific are involved.

Several investigators have reported that LPS is neutralized by a two-step process that involves disaggregation of the LPS by a serum protein followed by association with lipoproteins (10, 14, 16). On the other hand, Johnson et al. used LAL to isolate a single labile protein, not in the lippoprotein density range, which was able to irreversibly disaggregate LPS and reduce its toxicity (4). It is possible that either or both mechanisms may account for the neutralization we find.

The fact that plasma from normal blood donors varies so markedly in its ability to neutralize LPS raises the possibility that the susceptibility of patients to development of clinical manifestations from gram-negative infection might also vary on this basis. Frozen plasma screened for high neutralizing ability may also be useful in the prophylaxis of treatment of endotoxemia. Fresh-frozen plasma is occasionally used to replace coagulation factors in the treatment of disseminated intravascular coagulation induced by gram-negative infections. The use of units high in neutralizing ability might provide additional benefit in this setting by decreasing LPS-induced pathology.

We conclude that the inhibition of the effect of LPS on LAL by human plasma reflects detoxification of the LPS by plasma and that LAL can therefore be used as a biological assay to measure the ability of plasma to neutralize LPS. Used in this manner, LAL might be used as a tool to examine the susceptibility of patients to LPS and to purify a plasma component with endotoxin-neutralizing activity.

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